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Prospective Study of the Duration and Magnitude of Viraemia in Children Hospitalised During the 1996–1997 Dengue-2 Outbreak in French Polynesia

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The magnitude and duration of viraemia in children admitted to the hospital with dengue was studied during a dengue 2 outbreak in French Polynesia in 1996–1997. Forty-nine patients from whom at least 3 plasma samples were available were included in the study. Based on analysis of IgG-ELISA and haemagglutination inhibition assay, 21 of these were primary and 28 were secondary infections. According to World Health Organization criteria, 42 were dengue fever and 7 were dengue haemorrhagic fever. Virus was detectable by reverse transcription-PCR in all patients for at least the first 3 days of the onset of fever, but was never detected after the 6th day (mean duration = 4.4 days). Plasma virus titers ranged from 1.7–5.6 Log_{10} $TCID_{50}$/ml. A significant difference was not observed in the magnitude and duration of viraemia in patients with primary versus secondary infections. The severity of the illness, however, was correlated with both criteria. ***J. Med. Virol. 60:432–438, 2000.***

KEY WORDS: dengue; viraemia; primary infection; secondary infection; severity; virulence

INTRODUCTION

In the past two decades, dengue fever has become one of the leading causes of morbidity and mortality in tropical and subtropical areas throughout the world [Monath, 1994]. The dengue virus, a mosquito-borne member of the family *Flaviviridae*, circulates in nature as four distinct serological types DEN-1, -2, -3 and -4.

According to the World Health Organisation (WHO), infection by dengue virus causes a spectrum of illness ranging from classical dengue fever (DF) a self-limiting illness characterised by fever, headache, myalgia, arthralgia, and abdominal pain to a more severe form, dengue haemorrhagic fever (DHF). A clinical definition of DHF [WHO, 1986] requires the simultaneous presence of fever, haemorrhagic manifestations, thrombocytopenia and haemoconcentration. The principal pathophysiological hallmark of DHF is plasma leakage due to an increase in vascular permeability. The WHO classification further subdivides DHF into four grades of severity. Grades III and IV, characterised by circulatory failure, can become life-threatening because of profound hypovolemic shock (dengue shock syndrome).

The pathogenesis of DHF has been ascribed to the presence of enhancing antibodies that are acquired during a primary infection and lead to an increase of infected cells, and thereby an increase in viraemia, during secondary infections [Halstead, 1989]. This hypothesis is termed antibody-dependent enhancement (ADE). Differences in virus virulence, however, have also been postulated to be the primary risk factor in severe forms of the disease [Rosen, 1989; Murgue et al., 1997, 1998; Deparis et al., 1998a].

Dengue is the only mosquito-borne flavivirus that causes human disease in the Pacific Islands. In French Polynesia it has been common for only one serotype to be transmitted during epidemic and inter-epidemic periods. In August 1996, after 7 years of dengue-3 transmission, dengue-2 appeared in French Polynesia and subsequently throughout the South Pacific [Chungue et al., 1998]. Between August 1996 and April 1997, 14,500 suspected cases of dengue were recorded (Ministry of Health, French Polynesia) with peak transmission in February 1997; a single fatal case was reported [Deparis et al., 1998a]. During the same period, 1943 (45.9%) of 4228 suspected cases were confirmed by the Virology Unit of the Institut Malardé in Papeete.

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This report describes observations made on viraemia in plasma of children admitted to the hospital during this outbreak.

MATERIALS AND METHODS

Study Population

The study was carried out on sequential plasma specimens obtained from children admitted between August 1996 and March 1997 at the Centre Hospitalier Territorial in Papeete (Tahiti, French Polynesia). Consent for drawing blood was obtained from the parents.

Patients were classified clinically according to the WHO criteria [1986] as DF and DHF Grade 1–4 as follows:

DHF Grade I: no spontaneous haemorrhage
DHF Grade II: spontaneous haemorrhage
DHF Grade III: circulatory failure (hypotension or narrowing of pulse pressure, rapid and weak pulse, cold clammy skin and restlessness)
DHF Grade IV: profound shock (undetectable blood pressure and pulse).

Any patient with evidence of acute dengue infection who did not meet criteria for DHF was assigned a clinical diagnosis of DF. The date of onset of fever, as determined by questioning the parents, was defined as Day 1. The date of defervescence was the day that the temperature felt below 38°C.

Blood Samples

Samples were collected in citrated tubes on admission, and at daily intervals until discharge. For each sample, plasma was obtained after centrifugation and aliquots were kept frozen at −80°C until use.

Virological and Serological Study

Virological study. Dengue-2 infection was diagnosed from the first plasma sample by semi-nested Reverse Transcriptase-PCR (RT-PCR) for rapid detection and for serotype determination of the virus. For rapid detection, the method described by Chungue et al., [1993] was used with the consensus dengue D1 (5′-TCAATATGCTGAAACGCGCGAGAAACCG-3′) and D2 (5′-TTGCACCAACAGTCAATGTCTTCAGGTTC-3′) primers of Lanciotti et al. [1992] located on C and prM genes respectively (PCR products of 511 bp for all four dengue viruses). The dengue serotype was determined by a second amplification in which the D2 primer was replaced by dengue virus type specific primers, TS1 (prM gene), TS2 (C gene), TS3 (C gene) and TS4 (prM gene) of Lanciotti et al. [1992]. The size of the resulting DNA band was characteristic for each dengue virus type. The amplified products at predicted size for dengue-2 (119 bp) were demonstrated by staining with ethidium bromide after agarose gel electrophoresis.

If the first plasma sample of a patient was positive for dengue-2 virus, then this and subsequent samples were tested for determination of quantitative viraemia. In parallel, inoculation into C6/36 *Aedes albopictus* cells was carried out with the first plasma sample as described by Chungue et al. [1992].

Serological study. IgM capture enzyme-linked immunosorbent assay (IgM-ELISA) was carried out on the sequential plasma samples, using 4 serotype antigens respectively [Chungue et al., 1989a]. All subjects with titres <400 in all the four antigens were considered negative.

Immunological status (primary and secondary or multiple dengue infection) was determined by IgG-ELISA on the first and the last plasma samples using the 4 dengue serotype antigens respectively, described by Chungue et al. [1989b]. Samples were considered negative when reciprocal titre was <100.

As French Polynesia is free of other mosquito-borne viruses, patients who were IgG-ELISA positive in the acute plasma, and had a rapid and high increase of IgG titres in subsequent samples, were considered to have secondary infections. Patients who were IgG-ELISA negative in the acute plasma and remained negative, or had slightly increase of IgG titres (<400) in subsequent samples were considered as primary infections.

Haemagglutination inhibition (HI) antibody against the four dengue viruses [Chungue et al., 1989b] was measured for two patients because IgG was negative on the first sample but positive on the last sample. In these cases we considered that the infection was primary when HI on the first sample was negative (<10) but had less than a four-fold increase in the last sample.

Determination of Viraemia by Quantitative RT-PCR

Methods. RNA was isolated from 10 μl of plasma with the guanidinium thiocyanate-silica method described by Boom et al. [1990]. Elution of RNA was carried out with 15 μl of sterile water PCR grade (Boehringer Mannheim, Germany) and 0.4 U/μl of Rnasin (Promega, Madison, WI).

cDNA synthesis and amplification was combined in a single reaction vessel using 10 μl of eluted RNA in 30 μl of the following components: 1× PCR-reaction buffer (100 mM of Tris-HCl, 500 mM of KCl, pH 8.3 ; Boehringer Mannheim), 2.5 mM of $MgCl_2$, 400 μM of PCR Digoxigenin (DIG) labeling mix [(Boehringer Mannheim) containing 2 mM dATP, dCTP, dGTP, 1.9 mM dTTP and 0.1 mM DIG-dUTP], 50 pmol of each of specific D1 and D2 consensus dengue primers described by Lanciotti et al. [1992], 10 U of Rnasin, 6 U of AMV Reverse Transcriptase (Promega) and 2 U of *Taq* DNA Polymerase (Boehringer Mannheim). The reactions were allowed to proceed in a Hybrid thermocycler programmed to incubate for 1 hr at 42°C and then with 30 cycles of denaturation (94°C for 30 sec) primer annealing and extension (63°C for 1 min).

Amplified products were detected using the PCR ELISA DIG detection kit (Boehringer Mannheim). DNA was denatured at 100°C for 5 min and then chilled on ice. Ten μl of product were transferred with 200 μl of the consensus dengue biotin-labeled probe

(5′-biotin-ACTATCATGHGYGGYTCTCC-3′) located on prM gene (kindly provided by V. Deubel), at 10 nM, into a streptavidin-coated microtiter plate and then incubated at 37°C, (optimal temperature of hybridization for dengue-2, data not shown), for 3 hr. After washing, 200 μl of the anti-DIG-peroxidase working solution was added to each well and plates were incubated at 37°C for 30 min, then washed before adding 200 μl of enzyme substrate 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate (6)] (ABTS™). ODs were measured at a wavelength of 405 nm.

The negative control was obtained from pooled samples from four healthy French military volunteers. Each plasma was confirmed to be virus negative by RT-PCR and inoculation to C6/36 cells, as well as dengue IgM and IgG negative.

Positive control was a plasma obtained from a 30-year-old woman having dengue-2 infection (confirmed by inoculation in C6/36 cells, semi-nested RT-PCR and sequencing on the envelope gene) during this DEN-2 outbreak. This control enabled confirmation and reproducibility of the experiments.

The DEN-2 strain NG9427 was used to obtain external standards. The strain was amplified by 4 passages in mosquito cells (2 passages on C6/36 cells and 2 on AP61 cells), and then precipitated with Poly-Ethylene-Glycol. The titre, determined by the method of Reed and Muench [1983], was 7.6 Log_{10} $TCID_{50}$/ml. This stock solution was then diluted with negative control plasma to obtain 12 external standards with titres ranging from 1.5 to 6 Log_{10} $TCID_{50}$/ml (steps of 0.5 Log_{10} $TCID_{50}$/ml). The titres of the standards were next confirmed by inoculation into C6/36 cells. All 12 standards as well as positive and negative controls, were used in each assay.

The OD values of the external standards were measured after 5, 10, 15, 20, 30 and 40 min to determine the time of incubation of ABTS™. It was observed that the high titres standards reached un-interpretable values after 10 min of incubation, whereas the lowest standards had to be incubated more than 20 min for OD $\geq$ 0.2. For this reason we routinely measured OD for all the standards, controls and samples after 5, 10, 20 and 40 min of incubation and plotted a standard curve for all the standards for each incubation times.

Interpretation of the results. Among the 4 OD values obtained for each plasma sample at the four different times of incubation, samples were matched with OD between 0.2 and 1.6. To calculate samples titres, each selected value was then plotted on the corresponding standard curve obtained at the same time of incubation. Results were expressed in equivalent of Log_{10} $TCID_{50}$/ml. Viraemia was considered as positive for a titre $\geq$ 1.7 Log_{10} $TCID_{50}$/ml corresponding to the value of the negative control multiplied by 1.3.

Statistical Analysis

Instead of comparing the means viraemia per day for DF and DHF cases, and for primary and secondary infections, a linear regression statistical method was used [Seger, 1997]. This allowed us to take into account the temporal trends of viraemia levels during the disease and to compare the global trends of viraemia levels over time between DF and DHF and between primary and secondary infections.

To compare the magnitude of viraemia between DF and DHF, we first constructed a global adjusted curve for the overall dengue cases: the common curve. We next constructed 2 specific adjusted curves, one for DF cases and one for DHF cases. Then, using regression analysis we compared the adjustment of the global curve to the adjustment of the 2 specific curves [Laur et al., 1998]. The difference between DF and DHF was considered as significant ($P < 0.05$) if the adjustment of the 2 specific curves (DF and DHF) was better than the adjustment of the common curve (overall dengue cases).

The same analysis was used to compare the viraemia levels over time between the primary and the secondary dengue infections.

RESULTS

Children Enrolled

Between August 1996 and March 1997, 198 children were admitted to hospital for suspected dengue. Dengue was confirmed by the laboratory (virus detection or IgM detection) in 123 (106 DF and 17 DHF). There was no fatal case in our study population.

Among the 123 DEN-2 patients, 48 children in whom viral RNA was detected by RT-PCR in the first plasma sample, and for whom there were at least 3 sequential samples were selected. One child with only one plasma sample at Day 4, was also included in this study because of the severity of the disease (DHF Grade IV). The ages of selected cases ranged from 2.5 months to 15 years (median 84 months). Male to female ratio was 2.5. According to the WHO criteria, there were 42 DF patients and 7 DHF patients (1 Grade I, 3 Grade II, 2 Grade III and 1 Grade IV). Five patients, with moderate to severe shock but without haemoconcentration were classified as DF. Ten patients were admitted on the first day of fever, 17, 11, 9 and 2 on the second, third, fourth and fifth day respectively. None of the DHF cases were admitted on the first day of fever. The mean duration of hospitalisation was 6.3 days (median 5.5 days). The IgG-ELISA and HI assays indicated primary infections in 21 patients (20 DF and one DHF) and secondary infections in 28 patients (22 DF and 6 DHF). The mean duration of fever was 4.9 days (SD = 1.4) with a median of 5 days. The mean duration of fever was shorter in DF patients compared to DHF (4.8 ± 1.3 and 5.7 ± 1.5 days) as well as in primary dengue (4.2 ± 1.3 days) compared to secondary infections (5.4 ± 1.2 days).

Duration and Magnitude of Viraemia

One-hundred seventy-five samples obtained as serial daily blood samples during the early course of illness were examined. The validity of the quantitative RT-PCR method was confirmed by the reproducible values

TABLE I. Duration of Fever, Duration, and Magnitude of Viraemia in Hospitalised Patients Classified as DF and DHF and in Patients With Primary and Secondary Infections

Number of patients	Duration of viraemia (days)	Duration of fever (days)	Mean virus titres by day of illness (equivalent LOG_{10} $TCID_{50}$/ml)					
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Total (n = 49)	4.4 ± 0.9	4.9 ± 1.4	4.6 ± 0.5 (10)	4.3 ± 0.7 (25)	4.3 ± 0.7 (36)	3.4 ± 1.4 (43)	1.8 ± 1.7 (43)	0.4 ± 1 (18)
DF (n = 42)	4.3 ± 0.8	4.8 ± 1.3	4.6 ± 0.5 (10)	4.2 ± 0.7 (23)	4.3 ± 0.7 (34)	3.3 ± 1.4 (38)	1.6 ± 1.7 (37)	0.4 ± 1 (14)
DHF (n = 7)	5 ± 0.6	5.7 ± 1.5		5 ± 0.4 (2)	4.9 ± 0.1 (2)	4.3 ± 0.8 (5)	2.9 ± 1.5 (6)	0.6 ± 1.1 (4)
Primary (n = 21)	4.4 ± 0.8	4.2 ± 1.3	4.6 ± 0.3 (6)	4.1 ± 0.7 (14)	4.1 ± 0.9 (18)	3.3 ± 1.1 (19)	1.5 ± 1.6 (20)	0.3 ± 0.9 (7)
Secondary (n = 28)	4.4 ± 0.9	5.4 ± 1.2	4.5 ± 0.6 (4)	4.4 ± 0.7 (11)	4.6 ± 0.5 (18)	3.5 ± 1.6 (24)	2 ± 1.9 (23)	0.5 ± 1.2 (11)

Numbers in parentheses: N° of samples tested.

obtained with the positive control in each experiment (3.3 ± 0.2 equivalent Log_{10} $TCID_{50}$/ml, n = 11). Viraemia was assumed to be detectable on the first day of fever and to end on the last day that virus could be detected in the blood by RT-PCR. In 7 patients viraemia was still detectable in the last sample (1 on Day 4 and 6 on Day 5). For the remaining 42 patients, the duration of viraemia varied from 3 to 6 days with a median of 4 days (mean: 4.4 days, SD: 0.9). None of the patients had a viraemia of less than 3 days. The mean duration of viraemia was lower in DF patients than those with DHF (4.3 ± 0.8 and 5 ± 0.6 days respectively), but was identical in primary and in secondary infections (4.4 days) as shown on Table I. Virus titres ranged from 1.7 to 5.6 equivalent Log_{10} $TCID_{50}$/ml. Means values showed little change during the first 3 days of fever (Table I). Patients with DHF had higher mean viraemia titres from Day 2 to Day 5 than patients with DF. By contrast, the mean titres of viraemia were not very different in patients having primary versus secondary infections (Table I). Viraemia levels were compared over time between DF and DHF cases as well as between primary and secondary infections. Our statistical analysis did not demonstrate any significant difference in the viraemia levels of primary compared to secondary infections (Fig. 1). By contrast, however, there was a significant difference ($P = 0.047$) in viraemia of DF compared to DHF patients (Fig. 2).

As only one out seven DHF patients had a primary infection, it was not possible to assess the relationship between viraemia and severity of disease in patients classified as primary or secondary dengue.

Antibody Response

IgM antibodies were not detected on any day in 8 patients (4 primary and 4 secondary), for which the first and the last samples were IgM negative. Table II shows the day that IgM became detectable in the remaining 41 patients. The majority had IgM response from Day 4. In addition, 65% of the patients for whom IgM could be detected before Day 5 were primary dengue whereas 81% with IgM detection from day five were secondary. In all secondary cases, IgG was detectable before IgM (data not shown).

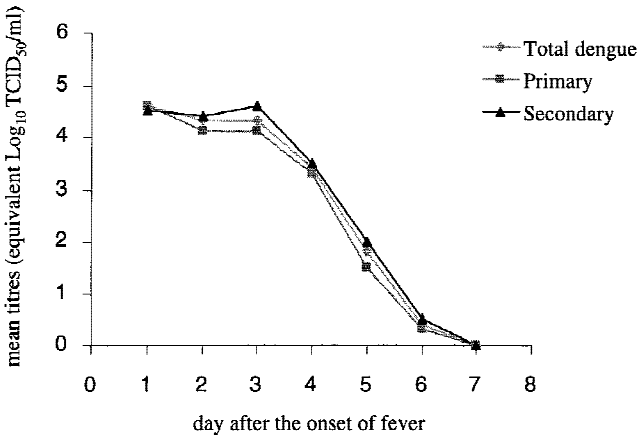

Fig. 1. Mean virus titres by day of illness in overall patients (♦) and in patients with primary (■) and secondary (▲) dengue. Number of samples tested are depicted in Table I. Mean virus titres were expressed as equivalent Log_{10} $TCID_{50}$/ml. The difference between primary and secondary dengue using a linear regression analysis was not significant ($P > 0.05$) because the adjustment of the two specific curves (■ for primary cases and ▲ for secondary cases) was not better than the adjustment of the common curve ♦.

DISCUSSION

Antibody dependent enhancement has gained wide acceptance as an explanation for the occurrence of severe dengue during the past decades. In the context of this hypothesis, viraemia is clearly an important aspect of dengue pathology, so it is surprising that it has not been extensively studied.

Studies that have reported the magnitude of dengue viraemia have indicated a variability in the amount of circulating dengue virus, according to serotype and even different strains of the same serotype. None have given evidence, however, of higher viraemia in DHF compared to DF [Kubersky et al., 1977; Gubler et al., 1979, 1981; Miagostovich et al., 1993], nor of higher viraemia in secondary infections compared to primary infections.

In all previous studies, quantification of viraemia was by the mosquito inoculation technique. This very sensitive method, however, requires 14 days to obtain results. By contrast PCR is a much more convenient

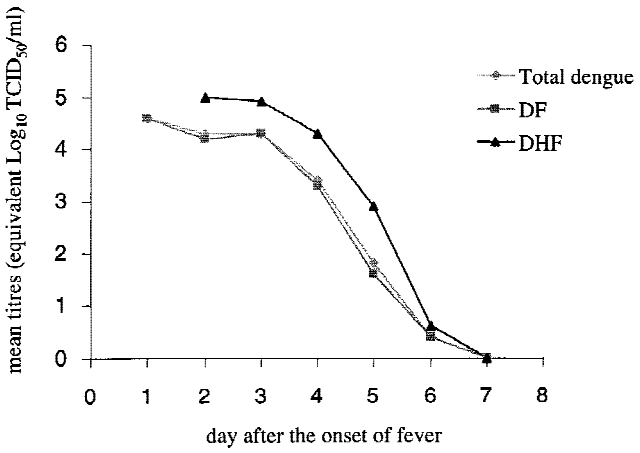

Fig. 2. Mean virus titres by day of illness in overall patients (♦) and in DF (■) and DHF (▲) patients. Number of samples tested are depicted in Table I. Mean virus titres were expressed as equivalent Log_{10} $TCID_{50}$/ml. The difference between primary and secondary dengue using a linear regression analysis was significant ($P < 0.05$) because the adjustment of the two specific curves (■ for DF cases and ▲ for DHF cases) was better than the adjustment of the common curve ♦.

technique as it does not depend upon growth of the virus. Chungue et al. [1993] demonstrated that RT-PCR for isolation of dengue virus RNA was a sensitive (detection limit as low as <2 Log_{10} $TCID_{50}$/ml), rapid and simple procedure for the diagnosis of dengue infections and in excellent agreement with C6/36 culture as well as with mosquitoes caught in the field. The quantification of dengue viral RNA in serum or plasma is a simplified procedure for assaying viral replication and has been applied to the quantitation of other RNA viruses.

For these reasons the RT-PCR procedure followed by hybridization of amplified products with a biotinylated probe and by ELISA were used to determine the duration and the magnitude of viraemia in serial daily plasma samples of 49 children hospitalised during the 1996–97 DEN-2 outbreak in French Polynesia. Most of the children enrolled in this study, were admitted to the hospital shortly after the onset of illness (78% between Day 1 and Day 3). In this study, viral RNA was always detectable up to the third day after the onset of fever but was not isolated after the sixth day. The magnitude of viraemia was lower than that reported in other studies [Gubler et al., 1981; Miagostovich et al., 1993] carried out in other dengue areas. This could be due to the relative mildness of this dengue-2 epidemic.

In our study population, viraemia was more persistent in DHF patients than in those with DF but did not differ in primary compared to secondary infections. These findings are not consistent with the data of Kuberski et al. [1977], Gubler et al. [1981], and Vaughn et al. [1997], who observed a longer viraemia in children experiencing primary infections. It was found in the current study that the magnitude of viraemia was not different between primary and secondary cases. We did find, however, that viraemia was significantly higher in patients classified as DHF compared to those classified as DF. Previous studies did not indicate any correlation between the magnitude of viraemia and the severity of the illness [Gubler et al., 1981]. Indeed, higher virus titres [Kuberski et al., 1977] or a slightly higher viraemia [Gubler et al., 1981] was observed in children experiencing primary infections. Taken together, the results indicate that, in our study population, the severity of the disease was correlated to the magnitude of viraemia, as it has been proposed for other viral infections [De-Moliner et al., 1998; Barker et al., 1998]. By contrast, no relationship was found between these criteria and history of previous dengue infection.

In the current study, dengue IgM developed later in secondary infection compared to primary infections, and was preceded by detectable IgG. These results are different with those of Vaughn et al. [1997] who observed that IgM antibody developed later in primary infections. They are in agreement, however, with the data of Innis et al. [1989] and Gubler [1996] who observed that despite variations in the kinetics of the IgM response in secondary infections, these antibodies appeared later than in primary infections, and that the detection of IgM was often preceded by IgG.

The understanding of dengue virus pathogenesis has been hampered by the lack of in vitro and in vivo models of the disease. The ADE hypothesis, however, substantiated by experimental infections of monkeys [Halstead et al., 1973] and by in vitro experiments [Marchette et al., 1976; Halstead and O'Rourke, 1977], has been proposed to explain the epidemiological observations made in the mid-60 in Southeast Asia. In these studies, it had been shown that most patients with DHF appeared to have had secondary dengue infection [Halstead and Yamarat, 1965].

In a recent prospective serological study, however, Deparis et al. [1998b] demonstrated that dengue incidence and dengue prevalence was dependent on time of exposure to the risk of receiving infected mosquitoes bites, and thus strongly on age in endemic and epidemic countries. Most epidemiological studies in dengue endemic regions have been conducted without adjustment for age, leading to the conclusion that DHF is more frequent during secondary than during primary infection.

Epidemiological studies in a number of geographic locations have linked DEN-2 in secondary dengue to the risk of DHF. In some studies, risk for DHF was associated with the DEN-1/DEN-2 infection sequence whereas in others it was the DEN-3/DEN-2 sequence [Sangkawibha et al., 1984; Burke et al., 1988; Hayes et al., 1988 ; Kouri et al., 1989; Guzman et al., 1990; Qiu et al., 1991].

Nevertheless, although the 1996–1997 dengue 2 outbreak in French Polynesia, followed a dengue-3 epidemic, it was remarkable for its mildness, in comparison to the 1989–1996 DEN-3 epidemic [Deparis et al., 1998a; Chungue et al., 1998], and this was despite the high proportion of secondary infections as described by

TABLE II. Developement of IgM Antibody in 41 Documented Patients

Number of patients	Day of illness							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Total (n = 41)	0	1	4	15	8	9	3	1
Primary (n = 17)	0	1	4	8	3	1	0	0
Secondary (n = 24)	0	0	0	7	5	8	3	1

Deparis et al. [1998b]. Although the proportion of secondary cases in the present study was 57%, in a recent report based on clinical and biological data for 403 laboratory-confirmed hospitalised dengue cases, we observed that disease severity was not related to immunological status [Murgue et al., 1999].

Finally, the results described above do not support the hypothesis that enhancing antibodies acquired during a primary infection favor the occurrence of DHF by increasing the magnitude of viraemia.

Other mechanisms, independent of the occurrence of secondary infections, may be involved in dengue severity, and this as a causal relationship between ADE and DHF remains unverified [Bielefeldt-Ohmann, 1997]. The results support previous findings [Murgue et al., 1997, 1998; Deparis et al., 1998a,b] that factors related to the virus itself (viral load and differences in viral virulence between dengue strains of the same serotype) play a dominant role in the severity of dengue.

It is considered that the severity of dengue infection may result from interactions between a particular virus and a particular vector, inoculated at a specific load in a particular host. Thus, to further understanding of the mechanisms of dengue pathogenesis, we suggest that more attention should be paid to viral factors, host-virus and vector-virus interactions.

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